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Adapter protein NRBP associates with Jab1 and negatively regulates AP-1 activity

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Abstract **Jun activation domain-binding protein 1 (Jab1) is a coactivator of activating protein-1 (AP-1) and is the fifth component of the COP9 signalosome complex. It interacts with a variety of proteins and plays important roles in diverse signaling pathways and cellular function including oncogenesis. We show here that Jab1 interacts in vivo with nuclear receptor binding protein (NRBP), an evolutionarily conserved adapter protein with a kinase-like domain. We further show that NRBP inhibits Jab1-induced phosphorylation of c-Jun and AP-1 activation. Finally, overexpression of NRBP in mammalian cells specifically inhibits AP-1 activation by various stimuli. Taken together, our data suggest that NRBP may be an important negative regulator of Jab1-mediated functions such as gene transcription and tumor progression.**

Keywords: NRBP; AP-1; Signal transduction; Jab1

1. Introduction

The COP9 signalosome (CSN) was originally identified in *Arabidopsis* as a negative regulator of photomorphogenesis [1]. In mammalian cells, the complex is composed of eight subunits and is involved in protein phosphorylation and degradation [2–5]. Each subunit of the CSN has been identified as the binding partner of many other proteins and thus has specific functions [6]. The precise biochemical functions of the CSN complex are still under intensive investigation. However, each subunit has been independently characterized [7–9]. Jun activation domain-binding protein 1 (Jab1), initially identified as a coactivator of AP-1 [10], is the fifth component (CSN5) of the CSN complex [11,12]. It was first identified through its interaction with the activation domain of c-Jun [10]. It was found to stabilize transcription factor complexes containing c-Jun or JunD at their AP-1 binding sites, leading to an increase in gene transcription of specific genes. In addition, Jab1 has also been shown to interact with a variety of proteins and control their functions, including regulation of gene transcription and cell cycle via phosphorylation, and degradation of target proteins [13]. For instance, interaction of Jab1 with the tumor suppressor p53 induces CSN-mediated phosphorylation and subsequent degradation of p53 [2]. Jab1 is also known to interact with the cyclin-dependent kinase inhibitor p27kip1 and decrease the stability of p27kip1, a process strongly associated with tumor progression [14]. A number of cellular factors and proteins including Vitamin D3 up-regulated protein 1 (VDUP1), migration inhibitory factor (MIF), and integrin LFA-1 have been shown to interact with Jab1 and modulate the Jab1-mediated functions such as AP-1 activity and cell proliferation [15–17].

NRBP is an ubiquitously expressed adapter protein containing a potential Src homology 2 (SH2) domain binding region, a kinase-like domain, and a myeloid leukemia factor 1 (Mlf1) binding region [18,19]. It represents a family of evolutionarily conserved proteins with homologs in *C. elegans*, *D. melanogaster*, mouse and human. Intriguingly, the kinase-like domain of NRBP, which contains significant homology with other kinase domains, lacks the conserved ATP-binding motif. To date, little is known about the function of NRBP except its interaction with a few cellular proteins. For example, the small GTPase Rac3 has been shown to form a complex with NRBP. However, overexpression of NRBP in mammalian cells failed to activate downstream targets of Rac3 including the JNK and the p38 pathway or associate with actin cytoskeletal rearrangements. Nonetheless, expression of NRBP led to significant redistribution of Golgi-associated marker p58 to the peripheral ER compartment, suggesting it may be involved in subcellular trafficking [20]. The highly conserved murine homolog of NRBP, MADM, has been shown to bind to Mlf1 oncoprotein, and mediate phosphorylation of the 14-3-3 binding site of Mlf1 [19]. Since MADM does not contain an ATP-binding motif in its putative kinase domain, it is hypothesized that the murine homolog of NRBP associates with an unknown serine-threonine kinase that phosphorylates Mlf1 and modulates its activity. In addition, given the presence of a nuclear localization and a nuclear export sequences in MADM, it may shuttle between the nuclear and cytoplasmic compartments, potentially regulating the subcellular localization and activity of Mlf1.

In this study, we employed a yeast-two hybrid screening and identified that a key gene activation and growth regulator Jab1

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Abbreviations: NRBP, nuclear receptor binding protein; Jab1, Jun activation domain-binding protein 1; AP-1, activating protein-1

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interacts with NRBP in vivo. Moreover, overexpression of NRBP in mammalian cells significantly impaired Jab1-mediated AP-1 activation. We further provided evidence that NRBP negatively regulates stimuli-induced activation of AP-1, but not NFκB. Our data strongly suggest that NRBP may be an important modulator in Jab1-mediated cellular function.

2. Materials and methods

2.1. Yeast two-hybrid screen

A yeast two-hybrid screen was performed using the Matchmaker GAL4 Two-Hybrid System 3 (Clontech). Full-length human NRBP cDNA was subcloned into pGBKT7 vector and the resulting bait plasmid was used to screen a pACT2 HeLa cell cDNA library (Clontech) following the manufacturer's protocol. A total of 7.5×10^5 colonies were screened and those that grew on selection plates were streaked on fresh plates. To eliminate false-positives, colonies were assayed for β-galactosidase activity using X-gal as the substrate.

2.2. Cell line and transfection

Human embryonic kidney (HEK) 293T cells were purchased from ATCC (ATCC: CRL-11268) and were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA) containing 10% FBS (GIBCO). Cells were transfected with indicated plasmids using the Lipofectamine™ 2000 reagent (Invitrogen) according to manufacturer's instructions. Simian virus 40 T antigen-transfected human leukemic Jurkat T (Jurkat-TAg) cells (ATCC TIB-152) were grown in RPMI 1640 medium (PAA, USA) supplemented with 10% FBS (GIBCO). Cells were transfected with indicated plasmids using the Superfect transfection reagent (QIAGEN).

2.3. Coimmunoprecipitation and Western blots

A glutathione *S*-transferase fusion protein consisting of the first 100 amino acids of NRBP was expressed in *Escherichia coli* using pGEX-6P-1 as previously described [19]. The fusion protein was purified, and antisera were raised in rabbits and subsequently purified. The cDNAs encoding wildtype and truncated human NRBP were subcloned into the pEF1a-promoter-driven expression vector in-frame with either an N-terminal Flag epitope followed by an IRES GFP. The full-length human Jab1 was subcloned into a pHA expression vector (Clontech) with an N-terminal HA epitope. HEK 293T cells were transfected with indicated plasmids. After 24 h, cells were harvested and lysed in 200 μl NP-40 lysis buffer (1% NP40, 150 mM NaCl, 10 mM Tris–HCl (pH 7.5), 5 mM EDTA and protease inhibitors, as previously described) [21]. Twenty microliter of lysates were used for western blot analysis to compare protein expression levels. For coimmunoprecipitation, 100 μl cell lysates were incubated with indicated antibodies at 4 °C for 1 h and with protein G-Sepharose beads (Sigma) for 2 h at 4 °C. After the beads were washed with lysis buffer, the bound proteins were eluted by boiling in SDS sample buffer, separated by SDS–PAGE, and electrophoretically transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were incubated with indicated primary antibodies followed by secondary anti-mouse IgG horseradish peroxidase (HRP) (Santa Cruz Biotechnology) and assayed by enhanced chemiluminescence (SuperSignal Western Blotting Kit, PIERCE). Coimmunoprecipitation of endogenous Jab1 with endogenous NRBP was performed with lysates of HEK 293T cells. Cell lysates were incubated with either the rabbit polyclonal anti-NRBP serum or the control preimmune serum at 4 °C for 2 h, followed by incubating with protein G-Sepharose beads (Sigma) for additional 2 h at 4 °C. Bound proteins were visualized by sequential Western blotting with the anti-Jab1 antibody and anti-NRBP antiserum.

2.4. Reporter gene assays

Approximately 1×10^5 293T cells were plated into each well of a 24-well plate 24 h before transfection. The indicated cDNAs with pAP1-luc reporter plasmid (Clontech) were cotransfected into cells. Empty vector was added to each transfection to keep the total amount of DNA constant. pRL-TK was used to standardize the efficiency of transfection. Cells were harvested 24 h after transfection, lysed in the reporter lysis buffer (Promega) and assayed using the Dual-Luciferase® Reporter Assay System (Promega). Luciferase activity was measured with a luminometer (Dynex Technology). For cell treatment, 293T cells were stimulated at 37 °C in the growth medium containing 40 ng/ml of PMA. After 8–12 h, cells were lysed and assayed as above. For Jurkat TAg cells, plasmids were transfected using the Superfect transfection reagent (QIAGEN). Cells were cultured for 40 h after transfection and aliquoted into a 96-well plate. They were stimulated at 37 °C in the growth medium containing 1 μg/ml anti-TCR hybrioma supernatant. After 8 h stimulation, cells were lysed and assayed as described above.

2.5. Antibodies

Mouse monoclonal anti-phospho-Jun (Ser-63) antibody, anti-Jab1 antibody and Goat anti-mouse IgG secondary antibody-conjugated with HRP were from Santa Cruz Biotechnology. Mouse monoclonal anti-Flag M2 (DYKDDDDK) antibody and anti-HA (hemagglutinin: YPYDVPDYA) antibody (12CA5) were obtained from Roche Diagnostics (Indianapolis, IN, USA). Polyclonal rabbit antiserum against human NRBP was generated against bacterially produced recombinant protein (Shanghai Genomics).

3. Results

3.1. NRBP interacts with Jab1 in yeast and in mammalian cells

To identify proteins that interact with NRBP, full-length human NRBP was used as the bait in a yeast two-hybrid screen against a Hela cDNA library. The initial screening identified four independent colonies that grew in the absence of Trp, Leu, and His. All of these colonies were further confirmed to possess β-galactosidase activity, and the subsequent sequence analyses identified two of the clones containing cDNA inserts corresponding to almost the entire coding sequence (amino acids 28–335; amino acids 49–335) of Jab1 cDNA. The identification of the two independent clones encoding overlapping regions of Jab1 provides strong confirmation of the specificity of the interaction between NRBP and Jab1 in our yeast two-hybrid assay.

Ectopic expression and coimmunoprecipitation assays were performed to further examine whether NRBP interacts with Jab1 in vivo. First, we cotransfected an HA-Jab1 with either a control vector or a Flag-NRBP in HEK 293T cells. As shown in Fig. 1A, NRBP associated with Jab1 when co-expressed. Furthermore, we investigated the in vivo interaction of endogenous partners expressed at normal levels. Endogenous NRBP was precipitated from untreated HEK 293T cell lysates with an anti-NRBP antiserum. Specific coimmunoprecipitation of Jab1 was detected by Western blot analysis with an anti-Jab1 antibody when the anti-NRBP serum, but not the control serum, was used for immunoprecipitation (Fig. 1B). Taken together, our data demonstrate a novel and specific interaction between NRBP and Jab1 in mammalian cells.

3.2. The N-terminal and the C-terminal regions of NRBP are both necessary for its binding to Jab1

To further characterize the NRBP-binding domain involved in the interaction with Jab1, we generated a number of deletion mutants of NRBP including NRBP (71–535), NRBP (40–535), NRBP (20–535), NRBP (1–406), NRBP (1–455), and NRBP (1–485), and examined their abilities to bind to Jab1. As shown in Fig. 2, full-length NRBP, NRBP (20–535), and NRBP (40–535) bound to Jab1, whereas NRBP (71–535) failed to bind to Jab1, suggesting that the region between amino acid 40 and 71

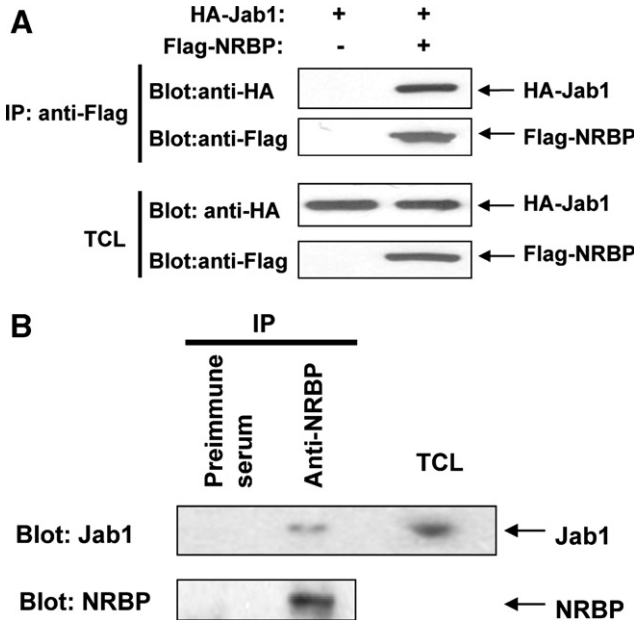

Fig. 1. NRBP interacts with JAB1 in vivo. (A). NRBP associates with Jab1 in mammalian 293T cells. HEK 293T cells were cotransfected with pHA-Jab1 and either pEF-Flag-NRBP or pEF-Flag control vector. Cell lysates were immunoprecipitated with anti-Flag (M2) antibody followed by immunoblotting with the corresponding antibodies. TCL: total cell lysates. (B). Endogenous NRBP was immunoprecipitated from HEK 293T cell lysates with the anti-NRBP antiserum and Jab1 was detected by immunoblotting with the anti-Jab1 antibody. Precipitation with the preimmune serum served as a control. The blot was also probed with the anti-NRBP antiserum (lower panel). Expression level of endogenous Jab1 in the total cell lysates (TCL) was determined by Western blotting with the anti-Jab1 antibody.

is important for this association. Interestingly, a C-terminal deletion mutant, NRBP (1–406) failed to interact with Jab1, indicating that regions in the C terminus of NRBP are also required for the interaction. Further deletion analysis showed that the very C-terminal region (residues between 406 and 455) was important in mediating the interaction, since NRBP (1–455) was capable of binding to Jab1. These data demonstrate that both the N- and the C-terminal regions of NRBP are involved in the Jab1 association.

3.3. Regulation of AP-1 activity by the interaction of NRBP with Jab1

The transcriptional coactivator function of Jab1 was initially characterized by its potent activation of AP-1 transcriptional activity [10]. Transient transfection assays in 293T cells have revealed that overexpression of NRBP had a moderate inhibitory effect on basal or c-Jun induced AP-1 activity (Fig. 3A). We therefore examined whether NRBP, through its binding to Jab1, might modulate the Jab1-mediated AP-1 activation in mammalian cells. 293T cells were transfected with an AP-1-driven luciferase reporter gene in the presence of either a control vector, c-Jun, or c-Jun together with Jab1. Overexpression of c-Jun increased the AP-1 activity, which was further potentiated by the coexpression of Jab1 (Fig. 3B). NRBP exhibited a marked inhibitory effect on the c-Jun and Jab1-mediated AP-1 activation in a dose-dependent manner. It has been well documented that AP-1 activation is a critical event in the activation and proliferation of many cell types including fibroblast and T cells. Moreover, a recent report has implicated Jab1-mediated AP-1 induction in proper T cell activation [17]. Consistently, we also observed a similar inhibitory effect of NRBP on the Jab1-induced AP-1 activation in Jurkat TAg cells in a dose-dependent manner (data not shown).

Interaction of Jab1 with c-Jun has been shown to increase phosphorylation of c-Jun, leading to the activation of AP-1 promoter activity [16]. To determine whether NRBP regulated the Jab1 function by affecting the level of phosphorylation of c-Jun, we then examined the levels of c-Jun and phosphorylated c-Jun in 293T cells in a similar cotransfection experiment as described in Fig. 3A. Consistent with previous findings, Jab1 induced c-Jun phosphorylation, and more importantly, NRBP inhibited this potentiation by Jab1 (Fig. 3C), These data suggest that NRBP not only physically interacts with Jab1 but also negatively modulates the Jab1-induced AP-1 transcription activity, likely through interfering Jab1-mediated c-Jun phosphorylation. Our data thus provide a novel insight into mechanism of action by which NRBP participates in cellular signaling pathways.

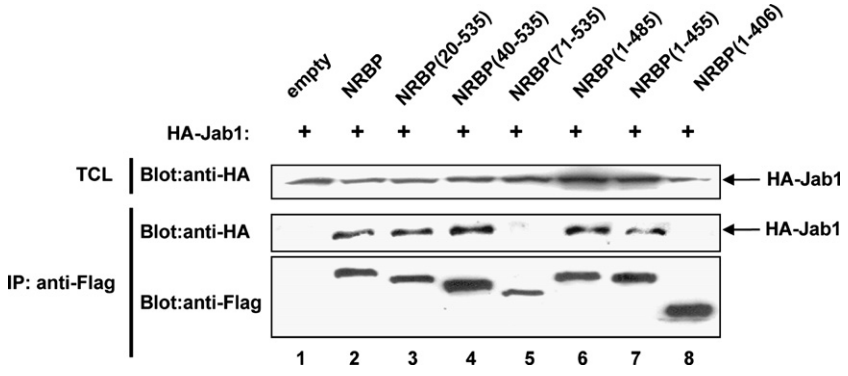

Fig. 2. Mapping the Jab1-interacting regions in NRBP. HEK 293T cells were cotransfected with pHA-Jab1 and the following plasmids: lane 1, pEF-Flag vector; lane 2, pEF-Flag-NRBP; lane 3, pEF-Flag-NRBP (20–535); lane 4, pEF-Flag-NRBP (40–535); lane 5, pEF-Flag-NRBP (71–535); lane 6, pEF-Flag-NRBP (1–485); lane 7, pEF-Flag-NRBP (1–455); and lane 8, pEF-Flag-NRBP (1–406). Cell lysates were immunoprecipitated with anti-Flag antibody followed by immunoblotting with the corresponding antibodies. Expressions of HA-Jab1 in the total cell lysates (TCL) were determined by Western blotting with an anti-HA antibody.

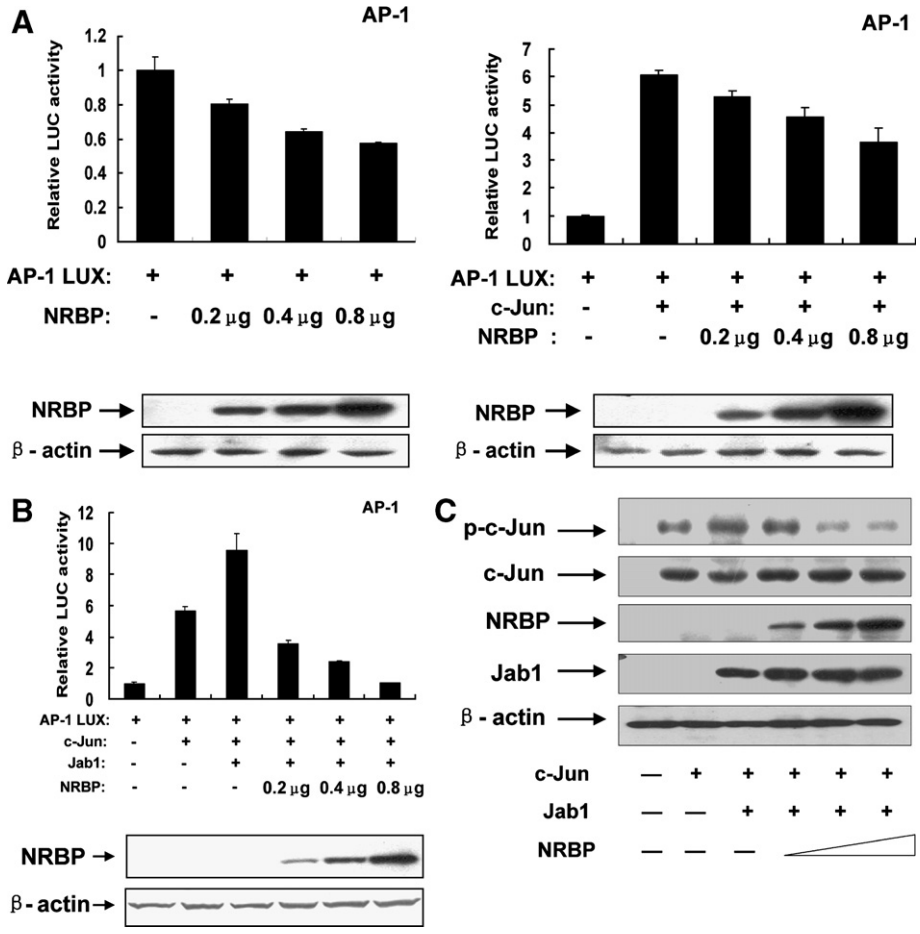

Fig. 3. Regulation of AP-1 activity by the interaction between NRBP and Jab1. (A) NRBP alone can interfere with basal or c-Jun induced AP-1 activity in HEK 293T cells. HEK 293T cells were transiently transfected with different amounts of pEF-Flag-NRBP (0.2, 0.4, and 0.8 μg), AP-1 luciferase reporter plasmid, and pEF-Flag-c-Jun or not. The total DNA concentration in each transfection was kept constant by addition with the empty vector. (B) NRBP inhibits the c-Jun and Jab1-mediated activation of AP-1 reporter activity in HEK 293T cells. HEK 293T cells were transiently transfected with pEF-Flag-c-Jun, pHA-Jab1, different amounts of pEF-Flag-NRBP (0.2, 0.4, and 0.8 μg), and the AP-1 luciferase reporter plasmid. The total DNA concentration in each transfection was kept constant by addition with the empty vector. (C) NRBP inhibits Jab1-mediated c-Jun phosphorylation. HEK 293T cells were cotransfected with the indicated cDNAs (designated as in (A)). The cell lysates were resolved on SDS-PAGE and analyzed by immunoblotting. The status of c-Jun phosphorylation was probed with an anti-phospho-c-Jun (Ser-63) antibody. The total amount of c-Jun, NRBP and JAB1 was probed with an anti-Flag or an anti-HA antibody, respectively. β-actin immunoblots served as a control.

3.4. NRBP inhibits stimuli-induced activation of AP-1, but not NFκB

To further investigate the specificity and physiological relevance of the negative regulation by NRBP, we examined the effects of overexpression of NRBP on stimuli-induced gene transcription in fibroblasts and in T cells. It is well known that many growth factors and pharmacological agents such as PMA activate AP-1 activity. We found that overexpression of NRBP also potently inhibited PMA-induced AP-1 activation in 293T (Fig. 4A) and Jurkat TAg cells (data not shown). It is also known that engagement of T cell antigen receptor (TCR) leads to AP-1 activation in T cells, a critical pre-requisite for T cell activation, cytokine secretion and proliferation. We showed that NRBP inhibited anti-TCR induced AP-1 activation in Jurkat TAg cells (Fig. 4B). More importantly, the inhibitory effect on AP-1 activation by NRBP appeared to be specific, since overexpression of NRBP completely failed to interfere with NFκB activation induced by PMA or a natural agonist TNF in 293T cells (Fig. 4C and D). Taken together, these results demonstrate that NRBP negatively regulates AP-1 activation in a specific and physiological manner, likely through its interaction with Jab1.

4. Discussion

NRBP is an ubiquitously expressed multi-domain adapter protein whose function so far remains largely elusive. Since its putative kinase domain lacks 7 of the 15 highly conserved amino acid residues in its catalytic site, it is unlikely to be possess enzymatic activity as a conventional kinase [18,20]. Other structural motifs in NRBP are two putative LXXLL nuclear receptor binding sequences. However, there is not yet any physical or functional evidence indicating that NRBP can bind to nuclear receptors. Interestingly, NRBP contains a bipartite nuclear localization sequence (NLS) and a nuclear export sequence (NES). Confocal microscopy and biochemical analyses revealed that NRBP was present in both the cytoplasm and nucleus, suggesting that it may shuttle between the compartments [22], and therefore it may regulate subcellular localization and

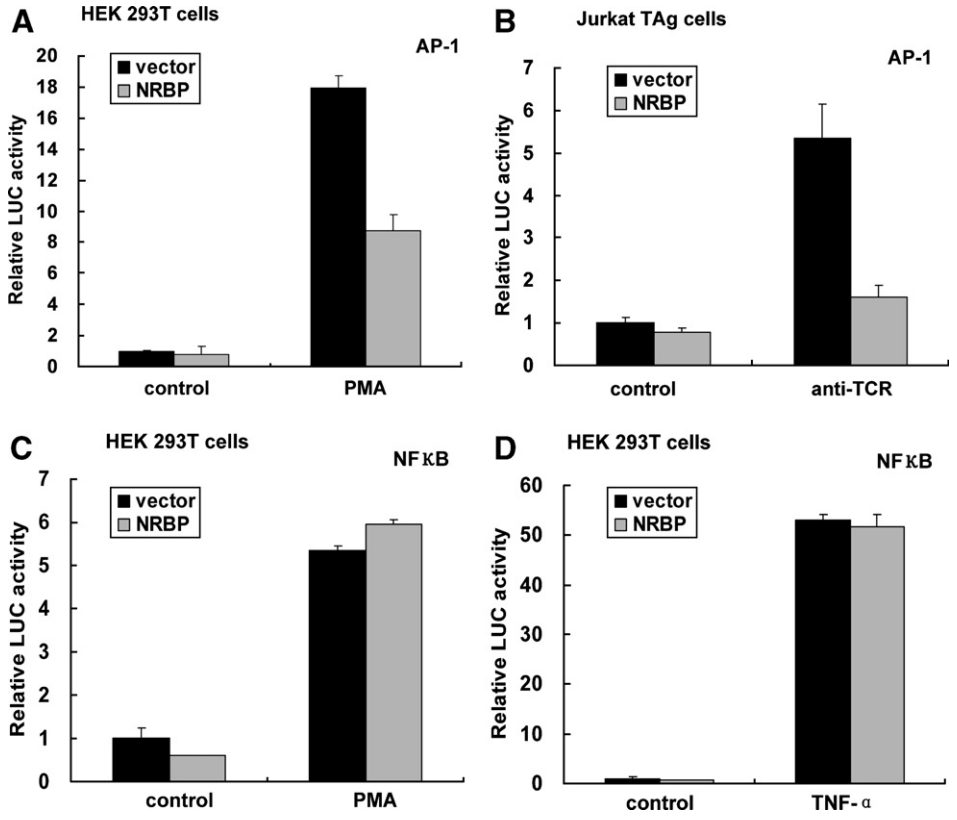

Fig. 4. NRBP inhibits stimulus-enhanced AP-1 activity. (A) NRBP inhibits PMA-induced activation of AP-1 in HEK 293T cells. Cells were transiently transfected with either pEF-Flag vector or pEF-Flag-NRBP, together with an AP-1 luciferase reporter plasmid. Cells were either untreated or treated with 40 ng/ml of PMA for 8–12 h. (B) NRBP inhibits anti-TCR enhanced activation of AP-1 reporter activity in Jurkat TAg cells. Cells were transiently transfected with either pEF-Flag vector, pEF-Flag-NRBP, together with an AP-1 luciferase reporter plasmid. Cells were either untreated or treated with 1 μg/ml anti-TCR for 8 h. (C, D) NRBP has no effect on the stimulus-enhanced NFκB activity. HEK 293T cells were transiently transfected with either pEF-Flag vector, pEF-Flag-NRBP, together with an NFκB luciferase reporter plasmid. Cells were either untreated or treated with 25 ng/ml PMA (C) or 10 ng/ml TNF-α (D) for 8–12 h.

activity of proteins involving in transcription signaling pathways.

In an attempt to explore potential function of NRBP in cellular signaling pathways, we searched for novel NRBP-interacting proteins using a yeast two-hybrid strategy. We identified Jab1, a key regulator for cell growth and gene transcription, binds to NRBP in vivo, and is negatively regulated by NRBP. Jab1 is a critical component of CNS and by far the most studied subunit [13]. CSN is a conserved protein complex which has now been found to participate in diverse cellular and developmental processes in various eukaryotic organisms. The CSN-associated protein kinase activity was initially described by Seeger et al. for the CSN complex purified from red blood cells [11]. In addition, the CSN preparation was found to phosphorylate c-Jun (Ser63 and Ser73), IκBα, and the NFκB precursor, p105, as well as tumor suppressor p53 (Ser149, Thr 150, and Thr 155) in vitro [2,11]. Jab1 can enhance the c-Jun phosphorylation level through a CSN-associated kinase and stabilize the binding of c-Jun to AP-1 sites [10]. Several reports have also implicated that an interaction between Jab1 and c-Jun contributes to Jab1's potentiation on AP-1 controlled transcription processes [16,17,23–25]. Jab1 is proposed to serve as a substrate docking site, and c-Jun binds via JAB1 to the CSN complex, leading to the phosphorylation of the amino-terminal c-Jun activation domain. Our data indicated that NRBP negatively regulates the level of phosphorylation of c-Jun enhanced by Jab1 or PMA, and subsequently down-regulates AP-1 activity induced by Jab1 or PMA. The inhibitory effect of NRBP on AP-1 activation likely was mediated through its interaction with Jab1, rather than affecting the upstream activators such as JNK, since overexpression of NRBP failed to interfere with phosphorylation of JNK [20]. It is also noteworthy that Jab1 has been implicated in influencing other transcription factors including NFκB through a different mechanism [26]. Indeed, our data demonstrated that NRBP specifically inhibited AP-1, but not the NFκB activation induced by various stimuli, suggesting that NRBP may primarily regulate Jab1-mediated signaling events leading to AP-1 induction.

Our deletion analyses revealed that both the N-terminus and the C-terminus of NRBP were required for its interaction with Jab1 in mammalian cells. Intriguingly, we found that the N-terminal region encompassing the first 99 residues was sufficient to interact with Jab1 in yeast (data not shown). It has been reported that the murine homolog of NRBP, Madm, interacts with the oncogene MLF1 through a region between residues 406 and 479 within Madm [19]. Since NRBP (1–455) was still capable of interacting with Jab1, it is unlikely that NRBP requires a binding partner like human MLF1 to facilitate its association with Jab1 [19]. We therefore hypothesize that the *C*-terminal NRBP may provide proper cellular localization or folding of the protein that allows its interaction with Jab1 in mammalian cells.

It is well known that AP-1 activation is a crucial prerequisite for cellular functions induced by a variety of stimuli, including T cell antigen receptor engagement. We found that overexpression of NRBP in T cells potently inhibited TCR-induced activation of nuclear factor of activating T cells (NFAT) (data not shown), in which AP-1 is an integral component. In addition, we showed that NRBP exerted its function more downstream of TCR stimulation, likely through Jab1, since other MAPK-mediated signaling events such as CD69 up-regulation was not affected by overexpression of NRBP (data not shown). Our data thus suggest that Jab1 may be an important player in TCR-induced signaling pathways leading to gene activation. In fact, a recent report has implicated Jab1's involvement in integrin LFA-1-mediated T cell activation [17]. It will be interesting to determine whether NRBP and/or Jab1 serve as a general modulator in T cell activation.

Abnormal AP-1 activation can also contribute to oncogenesis by affecting transcription of important regulators involved in cell proliferation, differentiation, apoptosis, and tumor invasion [27,28]. In addition, Jab1 has recently been strongly associated with aggressiveness or inferior overall survival in various malignancies including pituitary tumors, epithelial ovarian and breast cancer [29], and some types of lymphoma [30–33]. Therefore, our discovery of the physical and functional interaction between NRBP and Jab1 not only raises a possibility of NRBP's involvement in oncogenesis, but also provides additional insights into modulation of Jab1 and AP-1-mediated tumor pathogenesis.

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